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Modulation of Pathogenic B Cells through Inhibition of Phosphatidylinositol 3-Kinases

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Note: An abstract is required to be provided in Block 14

This proposal addresses the FY12 PRMRP topic area on lupus. Lupus is a life threatening disease that primarily affects women. Lupus patients develop antibodies that recognize proteins made by the body. This leads to tissue damage and complexes of the antibodies bound to the proteins can lodge in the kidneys resulting in damage to the filtering capacity of the kidney. The disease is most often managed using drugs that nonspecifically reduce inflammation and suppress the immune system. However that leaves the patient susceptible to other types of infections. Lupus treatment could be improved by specifically targeting the B cells involved in making the "self" antibodies. This proposal outlines one possible approach that could help solve this problem.

B cells express an important signaling molecule called PI3 kinase (PI3K). Activation of this enzyme leads to induction of survival pathways and is needed to promote development of antibodies. Therefore inhibition of PI3 kinase is expected to be beneficial for lupus by impairing survival of the pathogenic B cells and inhibiting their ability to produce antibodies. B cells express a specific form of PI3 kinase called delta. Small molecule inhibitors of the delta isoform have been shown to specifically kill B cell malignancies but leave other cells unaffected. Based on the success of the delta inhibitor in cancer research, it is anticipated that this approach will be particularly useful in lupus. Mice that are genetically predisposed to developing lupus will be treated with the PI3K delta inhibitor to determine if this ameliorates disease. If this works, it will provide an unprecedented level of control to target B cells and affect them by two mechanisms—survival and antibody production.

B cells also have survival mechanisms that work independently of PI3 kinase. One new lupus treatment is based on interfering with the other survival pathways. The drug, Benlysta, specifically neutralizes a B cell survival factor called BAFF (also known as Blys), which causes death of the mature antibody secreting B cells. Unfortunately it does not work for everyone and has shown little help among African-Americans. One possible reason is that the survival pathways mediated by PI3 kinase are capable to keeping many of the antibody producing B cells alive. Therefore, cultured B cells will be treated under conditions that mimic their interactions in the body to determine if interfering with both PI3K dependent and BAFF dependent survival results in even more B cell death than either approach alone. If true, experiments to test this in lupus prone animal models will be performed in the future.

Table of Contents

1. **INTRODUCTION:**

This project focuses on use of novel PI3kinase inhibitors to treat lupus. The PI3K/Akt pathway is a highly conserved pathway involved in numerous processes including survival. The immune system expresses a novel PI3K isoform referred to as delta, which plays a critical role in B cell signaling, antibody production, and survival. Recently PI3Kδ inhibitors have been developed to treat B cell malignancies. Since they target tumors that often have characteristics similar to antibody secreting B cells, we reasoned that a similar approach may be useful for treating lupus, a disease resulting in production of antibodies recognizes "self" components, such as nuclear proteins and DNA. These antibodies can cause additional pathologic changes because immune complexes lodge in the kidney which results in further damage. These experiments will test the hypothesis that PI3Kδ inhibition will reduce the frequency of pathogenic antibody (anti-dsDNA) secreting B cells in a mouse model for lupus, which results in less kidney damage and increased lifespan.

2. **KEYWORDS:** Lupus, PI3K, B cell, signal transduction

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Final Progress Report for PI3K inhibitor on Lupus

This project had received a no cost extension to allow us to complete the survival studies and final analyses. This will be the final report. It will incorporate the previous work presented in the Year 2 annual report as well as the newer data that we have generated since the annual report was submitted earlier this year.

Aim 1. Evaluate whether inhibition of PI3Kδ ameloriates the clinical pathology of lupus in NZB/NZW F1 mice

Aim 1. Our initial animal studies were designed to begin assessing the impact of treating lupus diseased mice with CAL101, a PI3Kδ inhibitor. Previous work by others showed that inhibition of PI3Kδ was a promising treatment for some classes of B cell malignancies. In fact, this class of inhibitors has been recently approved for mantle cell lymphoma. Tonic B cell receptor (BCR) signaling is needed for B cell survival and BCR signaling in general leads to antibody and cytokine production. While B cells express multiple isoforms of PI3K, BCR signaling is highly dependent on delta isoform. We reasoned that inhibitors of PI3Kδ would lead to reduced antibody production and should decrease the number of activated B cells. This would be expected to reduce the amount of disease in lupus.

Overview

During the first year of this project, we established an approximate timeline when we could detect pathogenic anti-dsDNA antibodies in the NZB/NZW lupus mouse model. We also showed that a number of cellular hallmarks associated with lupus were reduced after 1 month of treatment with an inhibitor of PI3Kδ. Using flow cytometry, we showed reduced germinal center B cells (B cells that differentiate into antibody secreting cells) and Tfh (T cell subset that promotes germinal center B cells. Most of these results were summarized in the annual report for year 1. These promising data suggested that this treatment approach might be effective in controlling lupus.

During the period covered by this annual report, we extended the above studies to determine differences in survival between treated and untreated mice. We took monthly serum samples during the survival study to evaluate the levels of circulating pathogenic antibodies. During year 1 we stored spleens and kidneys from mice treated with drug for one month in order to perform histology during year 2. We also collected these organs at termination of the survival study to compare results. Kidneys are a site of damage caused by lupus; pathogenic antibodies get lodged in the glomeruli and elicit complement mediated lysis of the cells. The damage results in proteinurea. Histological sections from these tissues are then stained to determine if there were changes in splenic germinal centers and antibody deposition in the kidney.

Results

To mimic a therapeutic approach for evaluating the potential of blocking lupus with PI3Kδ inhibitors, we began dosing mice only after they showed elevated titers of anti-dsDNA antibodies. For the survival studies, four month NZB/NZW mice had increased levels of pathogenic antibodies and began receiving twice daily doses of 10 mg/kg CAL120 (idelalisib), a PI3Kδ inhibitor that was recently FDA approved for treating some types of B cell leukemias. Blood was collected after 2 weeks of treatment to measure IgG and anti-dsDNA Ab. After one month of dosing, a cohort of mice were euthanized and spleens, blood, bone marrow, and kidneys isolated for further analysis. In addition, as mice were terminated during the survival portion of the study, blood and above organs were collected for further analysis.

Survival. The NZB/NZW mouse strain has a shortened lifespan due to tissue injury caused by immune complex deposition. In particular, a key site is kidney damage caused by lupus nephritis. Mice were dosed orally twice daily with vehicle or CAL120 (PI3Kδ inhibitor) and survival recorded. At study termination, all the vehicle treated mice had died or required euthanization due to high levels of proteinuria (evidence of kidney damage). In contrast there was 100% survival in the cohort receiving inhibitor (Fig 1).

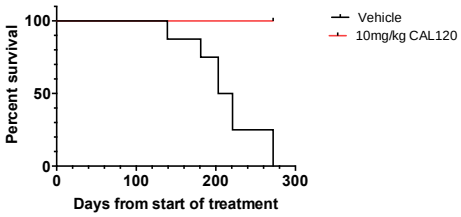

Fig 1. Treatment with PI3Kδ inhibitor improves survival of NZB/NZW mice. Mice began treatment at time of elevated anti-dsDNA antibodies (~4 months of age) and continued until vehicle group required euthanasia. No deaths occurred in CAL120 (inhibitor) treated mice.

IgG serum titers. A typical mouse has serum IgG concentration of ~5 mg/ml. The NZB/NZW mice develop hypergammaglobulinemia as disease progresses. We initiated treatment when IgG titers were elevated. There was no discernable difference in IgG levels between vehicle and CAL120 after two weeks of dosing (not shown). In contrast, after 4 weeks of treatment, the level of total IgG decreased and was statistically significant relative to vehicle alone. Since PI3Kδ inhibition has the potential of blocking all antibody production, we also examined IgG levels throughout the study to determine whether the circulating antibody levels were compromised. After 20 weeks on drug, the concentration of IgG reached a steady state level of ~5 mg/ml, which is considered the normal range in mice (Fig 2). This is suggestive that the drug does not completely inhibit antibody production, which distinguishes it from several treatments that result in B cell depletion. It may have a disproportionate effect on autoantibody production without affecting overall circulating antibody levels; this may prove beneficial in maintaining the ability to fight infections.

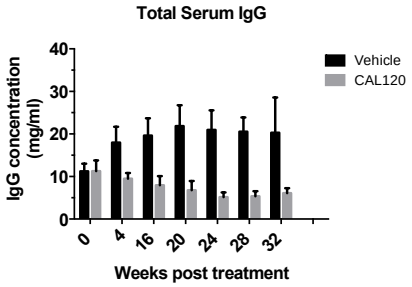

Fig 2. Reduction of hypergammaglobulinemia by treatment with PI3Kδ inhibitor. Mice began receiving inhibitor once IgG titers were increased over pre-symptomatic mice (~4 months). The IgG titers continued to increase, then plateaued, until the mice were ~8 months. In contrast, a decrease of total IgG was evident after 4 weeks on drug and continued to decline until reaching a steady state level of ~5 mg/ml (normal mouse range) around 20 weeks of treatment.

Anti-dsDNA titers. A hallmark of lupus is the production of autoantibodies against nuclear constituents, including dsDNA. We measured serum titers of anti-dsDNA in mice treated with either vehicle or the PI3Kδ inhibitor (Fig 3). The inhibitor prevents the spike in pathogenic anti-dsDNA antibodies and leads to a decrease in pre-existing levels as well, suggesting that this approach may be capable of managing the disease.

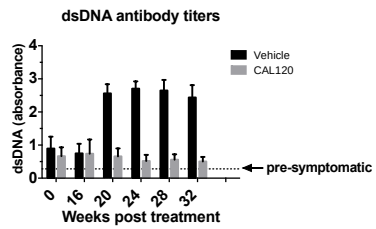

Fig 3. PI3Kδ inhibition prevents accumulation of circulating anti-dsDNA antibodies. Mice were initially screened for presence of anti-dsDNA antibodies. Animals with elevated levels were treated with inhibitor and serum collected monthly, then anti-dsDNA antibodies measured by ELISA. The level of antibodies in a young presymptomatic mouse is indicated for comparison.

Antibody deposition in kidney. Many patients with lupus develop lupus nephritis, which is caused by antibody complexes getting lodged in the kidney glomeruli. This allows for complement fixation, which results in damage to the glomeruli. Over time, the glomeruli no longer filter appropriately and the urine contains large amounts of protein. During the survival study, we monitored monthly changes in proteinuria. Animals that received the inhibitor only had trace levels of protein in the urine, whereas vehicle treated mice eventually had ~500mg/dL protein in the urine (Fig 4).

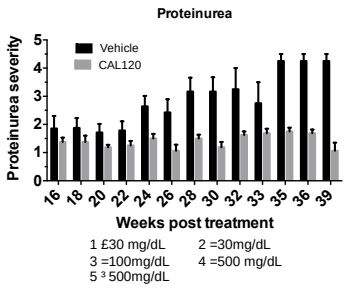

Fig 4. PI3Kδ inhibition protects against proteinuria. Mice treated with the PI3Kδ inhibitor showed scores of trace or 1. Over time vehicle treated mice developed severe proteinuria, with a maximal score of 4 (~500 mg/dL).

We also monitored IgG deposition in the kidneys (Fig 5). While most glomeruli from mice treated with vehicle showed Ig deposits, this was strongly reduced after only 4 weeks on the inhibitor (not shown). This result suggests the possibility of reversing or preventing further advancement of lupus nephritis. We then analyzed the distribution of IgG and complement C3 (C3) in kidneys during the survival study. Mice were treated with vehicle or PI3Kδ inhibitor for up to 9 months. Complement C3 is normally distributed in the interstitial space between glomeruli. During lupus nephritis, the C3 binds immune complexes and relocalizes inside the glomeruli. The resultant complement fixation leads to glomerular damage. Treatment with the PI3Kδ inhibitor prevented C3 from redistributing to glomeruli and probably explains why the drug treated mice do not develop proteinuria.

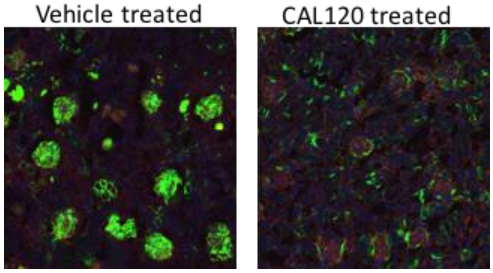

Fig 5. Glomerular immune complexes are reduced following treatment with PI3Kδ inhibitor. Kidneys were sectioned and stained with anti-IgG (red) and complement C3 (green). Complement normally localizes around the periphery (interstitia) of the glomerulus. It moves into the glomeruli in the presence of immune complexes. Note the drug treatment prevents the redistribution of C3, minimizing tissue damage.

Splenic changes in NZB/NZW mice treated with PI3Kδ inhibitor. The NZB/NZW mice develop splenomegaly as disease progresses. This is accompanied by an increase in germinal centers, which are sites where B cells differentiate into antibody secreting cells. Over time, the size of germinal centers also increases in the lupus prone mice. After 2 weeks on the inhibitor, the spleens showed a trend towards becoming smaller. The reduction in splenomegaly became apparent after 4 weeks on the drug and was statistically significant (Fig 6). The spleen size continued to increase in vehicle treated mice, whereas mice receiving drug showed a consistent size throughout the remainder of the study.

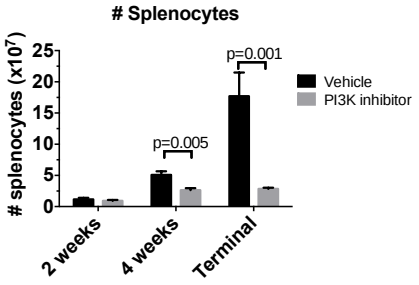

Fig 6. Inhibitor reverses splenomegaly associated with lupus. After 4 weeks treatment spleen size was reduced and remained constant in the presence of drug. Mice receiving vehicle showed a steady increase in spleen size over time.

We also examined the presence of germinal centers. In presymptomatic NZB/NZW mice, germinal centers (GC) are rare and small. In contrast, as disease progresses, the number of germinal centers increases markedly (Figs 7 and 8). The germinal centers are identified by PNA staining (green) and are located inside the B cell follicles (marked by IgD staining). In concert with the reduction in splenomegaly, the frequency of germinal centers also decreased by treatment with the PI3Kδ inhibitor and reached statistical significance after 4 weeks of treatment. Notably the number of B cell follicles was unaffected by the drug.

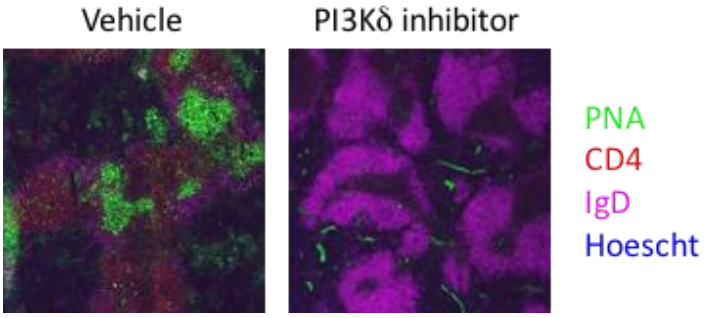

Fig 7. Germinal Centers (GC) are reduced after treatment with PI3Kδ inhibitor. Spleens were sectioned and stained with markers indicated on panel. The reduction in GC is consistent with reduction in pathogenic antibodies. PNA: marks germinal centers; IgD: marks B cell follicles; CD4: marks CD4 T cells, possibly Tfh

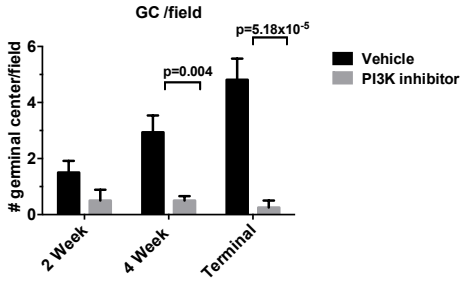

Fig 8. Quantitation of germinal centers (GC) in spleen. The number of PNA+ germinal centers (sites of antibody producing B cells) were counted in 10 fields from 5 spleens and average number of GC plotted. Germinal center frequency decreased after 2 weeks of treatment and remained reduced in mice receiving the inhibitor.

Frequency of immune subsets in spleen. Induction of GC B cells requires instruction from Tfh cells, a specialized subset of T cells found in the B cell follicle. Maintenance of both Tfh and GC B cells are dependent on each other. In the absence of an immune stimulus, Tfh cells are ~0.1% of the T cell population; following immunization the frequency increases to 1-5%. It has been previously reported that Tfh cells require PI3Kδ activity as well. We investigated the percentage of Tfh and GC B cells by flow cytometry in the presence or absence of drug. The percent of Tfh in presymptomatic NZB/NZW mice was approximately 0.2% of the splenic CD4 T cells (not shown); once disease was evident, the Tfh frequency had increased to 7% of the

splenic population (Fig 9). Within 2 weeks of treatment with PI3Kδ inhibitor, the number of Tfh had been reduced by half; by 4 weeks they represented ~1% of the CD4 T cell population. Notably, long term treatment reduced the number of Tfh to ~0.1%, the level found in unimmunized mice. Mice receiving vehicle showed a steady increase in Tfh numbers. The fact that the Tfh frequency can be reduced to background levels in inhibitor treated mice raises the intriguing possibility that it might be possible to "reset" the system and dramatically slow the recurrence of pathogenic antibodies.

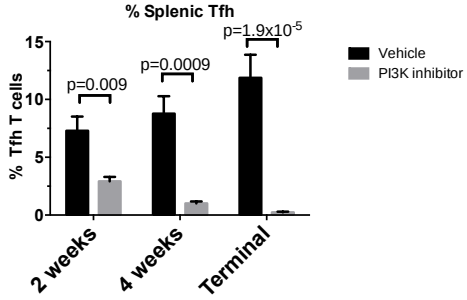

Fig 9. Reduction of Tfh cells by treatment with PI3Kδ inhibitor. Mice received vehicle or CAL120 (inhibitor) after dsDNA antibodies were detected. Note that Tfh cells decreased after as little as 2 weeks treatment and continued to drop with drug treatment.

We also quantitated the frequency of GC B cells by flow cytometry. As was observed by histology, the frequency of GC B cells was highly reduced by treatment with the inhibitor (Fig 10). Diseased mice had elevated levels of GC B cells, which was reduced with as little as 2 weeks treatment with the drug. Over time, the frequency of GC B cells increased in vehicle treated mice, but remained low in mice receiving CAL120.

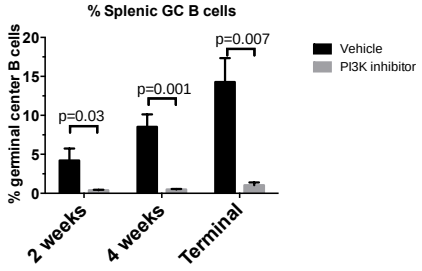

Fig 10. Germinal Center B cells are reduced following treatment with PI3Kδ inhibitor. Mice received vehicle or CAL120 (inhibitor) after dsDNA antibodies were detected. Note that GC cell numbers decreased after as little as 2 weeks treatment and continued to drop with drug treatment.

We also examined general B cells subsets. Previous work indicated that innate-like B cells such as marginal zone (MZ) B cells tend to be especially sensitive to PI3K activity. As expected, the frequency of this B cell subset decreased following treatment with the PI3Kδ inhibitor (Fig 11). We also noted that the number of class-switched B cells (those that have responded to antigen) was decreased with treatment. This may be due to the reduction in GC B cells, which are the subset that undergo class switching to produce antibody secreting cells.

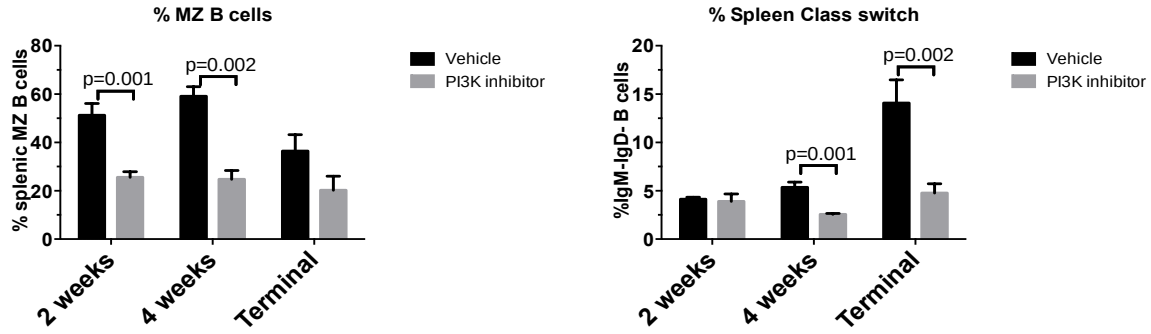

Fig 11. Frequency of B cell subsets affected by PI3K inhibition. Treatment with inhibitor reduces MZ B cells and cells undergoing class switching. The proportion of MZ B cells was reduced in as little as two weeks treatment. Changes in the frequency of B cells undergoing class switching (typically B cells that are responding to antigen) was statistically different after 4 weeks dosing.

In summary, treatment with the PI3Kδ inhibitor reverses emergence of potentially pathogenic immune subsets and improves survival. Together these studies suggest that use of the inhibitor may prove efficacious in treating this disease in humans.

Aim 2. Determine if simultaneous inhibition of PI3K and BAFF synergistically impairs survival of pathogenic B cells.

Aim 2. These experiments were summarized in the annual report for year 1. They are presented here again as part of the final report. Lupus patients have higher levels of circulating BAFF, a B cell survival factor. It has been hypothesized that the combination of BAFF and chronic B cell activation maintains B cells and they do not undergo apoptosis as readily, so the circulating pool of pathogenic B cells stays elevated. This has lead to a new treatment using anti-BAFF. Although not everyone improves with this treatment, it shows some evidence of efficacy in select populations.

Since BCR induced survival/proliferation relies heavily on PI3Kδ activity, we reasoned that inhibiting this isoform would reduce proliferation. However, since BAFF is elevated in lupus patients, it was unclear whether it could confer a survival advantage in the presence of the PI3Kδ inhibitor. To begin addressing this, we used in vitro cultures where we activated B cells through the BCR in the presence/absence of BAFF and CAL 101, a PI3Kδ inhibitor. Although CAL101 has been used to block in vitro proliferation, those reports used 1 uM of drug, which we found to be toxic. We were able to use as little as 10 nM CAL101 and obtain clear inhibition of proliferation. The BCR was activated by cross-linking with anti-IgM. Inclusion of 5 ng/ml BAFF increased overall proliferation ~3-fold, probably by preventing apoptosis. The presence of CAL101 blocked nearly all the proliferation induced by anti-IgM, whereas BAFF was able to only marginally improve proliferation. These results suggest that inhibiting PI3Kδ reduces B cell proliferation and probably survival, even in the presence of BAFF. Furthermore it lends credence to concept that PI3Kδ blockade may be a viable therapeutic option for lupus. Although beyond the scope of this grant, it would be of clinical interest to combine CAL101 with anti-BAFF treatment. If there is synergy, it may be possible to have lupus patients receive intermittent treatments rather than being chronically exposed to these drugs.

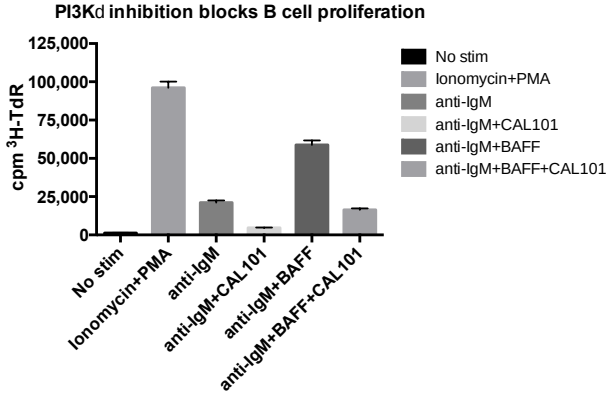

Fig 12. PI3Kδ inhibition reduces BCR induced proliferation. Purified B cells were plated in triplicate in microtiter dishes in the presence of 100 μg/ml anti-IgM and 5 ng/ml BAFF. After 3 days, 1μCi ^{3}H-thymidine was added and cells harvested the next morning. Ionomycin/PMA was used as a positive control. Note that CAL101 markedly reduces proliferation.

Since production of germinal center B cells was so profoundly affected by PI3Kδ inhibition in vivo, we also tested the hypothesis that inclusion of inhibitor would prevent in vitro differentiation of antibody secreting cells (ASC). Naïve B cells were stimulated in culture with IFNα and CD40L, then plated in ELISPOT wells to determine the frequency of antibody secreting cells. Doses as low as 125 nM inhibitor was sufficient to completely block development of the ASC. This established another route by which PI3Kδ inhibition can amelioriate the disease symptoms found in lupus.

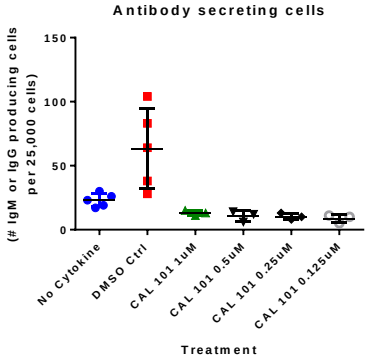

Fig 13. Inhibition of PI3Kδ blocks antibody secreting cell development in vitro. Cultures were treated with IFNα and CD40L to induce differentiation. Inclusion of CAL101 inhibitor prevented formation of ASC.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

Key Research Accomplishments:

- Treatment with the PI3Kδ inhibitor results in rapid reduction in hypergammaglobulinemia that is associated with lupus. Maximal response appears after 4 weeks of treatment
- Levels of pathogenic dsDNA antibodies highly reduced within 4 weeks of treatment
- Antibody deposits in kidney are reduced following treatment. Suggestive that this treatment has potential to reverse kidney damage associated with lupus nephritis
- The inhibitor prevents relocalization of complement C3 to glomeruli, sparing kidney damage caused by complement fixation.
- Splenomegaly and germinal centers (sites of B cells developing into antibody producing cells) in spleen reduced to levels observed in non-lupus prone mice.
- Longer term treatment with the inhibitor reduces the Tfh population to background (levels present in a non-immunized, non-lupus prone mouse), suggesting that the reduction of Tfh population may dramatically slow the recurrence of pathogenic germinal center B cells.
- The PI3Kδ inhibitor prolongs the lifespan of the NZB/NZW mice and prevents development of kidney disease as exemplified by lack of proteinurea and no immune complex deposition (marked by complement C3 staining) in the glomeruli.

5. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The data accumulated during this project demonstrates that inhibition of PI3Kδ can have a significant impact on reversing the symptoms and correlates of SLE. Based on these results, treatment with the PI3Kδ inhibitor is expected to hold much promise for treating lupus. During as little as 4 weeks of treatment, virtually all the markers associated with SLE were reversed. We showed that the elevated amount of IgG and pathogenic anti-dsDNA antibodies were reduced to levels typically seen in non-lupus prone mice. Moreover there was a survival benefit in lupus prone mice treated with the inhibitor. In all likelihood this was due to the prevention of lupus nephritis that is typically caused by immune complex deposition in the kidney glomeruli. Notably, lupus nephritis is a significant morbidity factor in humans. Consistent with this is the observation that Ig deposits in the kidney are reduced during treatment and complement C3 maintains its interstitial localization around the glomeruli, thus sparing kidney function.

Since lupus is a chronic disease, the safety profile for new treatments must be very high since patients will be taking drugs for a long time. Currently the PI3Kδ inhibitors are approved for cancer treatment, where there is more leeway in terms of acceptable safety. To limit exposure to the drug, one approach that might be worth considering is to dose mice for 4 weeks, then remove drug to determine how much time needs to elapse before there is an increase in pathogenic (anti-dsDNA) antibodies. If one round of treatment allows the mice to remain symptom-free for several months, treatment could be limited to a few times per year, which may lead to a better safety profile.

Targeting PI3Kδ is thought to predominantly affect the B cell compartment. However at least one report suggests that the delta isoform promotes Tfh development. Thus the efficacy of the inhibitor could be derived from the concerted effect on both the B cell and Tfh, which drives the germinal center response. Lupus is associated with a IFN signature; early trials using anti-IFN have shown some reduction of disease, suggesting that part of the pathology is due to the IFN axis. Previous work demonstrated that PI3Kδ inhibition blocks TLR9 induced IFN production by pDC, the cell type that is the predominant source of IFN. It may be possible to dissect the relative contribution of these cell types using more selective B cell inhibitors, such as those targeting Btk, a kinase needed for BCR signaling and B cell survival. Moreover, the selectivity of the Btk inhibitor (more B cell specific) conceivably may have fewer side effects which could be more permissive for use in chronic illnesses.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

   a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:
(2) Peer-Reviewed Scientific Journals:
(3) Invited Articles:
(4) Abstracts:

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
    a. Presentation is scheduled for May 2016 at American Association of Immunologists annual meeting in Seattle Washington.

7. **INVENTIONS, PATENTS AND LICENSES:** List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

   Nothing to report

8. **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.
   Nothing to report

9. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.
   Nothing to report

   For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

10. **REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

11. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and

professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on this eReceipt System https://cdmrp.org/Program_Announcements_and_Forms/ and under "Forms" on https://www.usamraa.army.mil) should be updated and submitted with attachments.

MARKING OF PROPRIETARY INFORMATION: Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the GOR to obtain approval. REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE. It is the responsibility of the Principal Investigator to advise the GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release."

– Data and Software Requirements" and https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information.